

ARTICLE

TLR9 agonist adsorbed to alum adjuvant prevents asthma-like responses induced by *Blomia tropicalis* mite extract

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Abstract

Blomia tropicalis mite is highly prevalent in tropical and subtropical regions and it is associated with allergic diseases such as rhinitis and asthma. By using an OVA-model of allergic lung disease, we have previously shown that sensitization in the presence of toll like receptors (TLRs) agonists attenuates subsequent OVA-induced allergic responses. Here, we evaluated the effect of CpG-ODN, a specific synthetic TLR-9 agonist, on the development of experimental asthma induced by *Blomia tropicalis* extract, a relevant source of aeroallergens. Among different protocols of *Blomia tropicalis* extract sensitization, the subcutaneous sensitization in the presence of alum adjuvant induced the highest Th2 responses, including high IgE levels. Adsorption of CpG to *Blomia tropicalis* extract/Alum attenuated the airway hyperreactivity, the infiltration of inflammatory cells including eosinophils, and the IL-5 content in BAL. In addition, lung peribronchial inflammatory infiltrate, mucus production and IL-5-producing CD3⁺CD4⁺ T cells were significantly reduced in the *Blomia tropicalis* extract/Alum+CpG group. Importantly, CpG inhibited total IgE production as well as active systemic or cutaneous anaphylaxis reactions. Inhibition of pulmonary Th2 responses was associated with increased IL-10 production but not with IFN- γ production. Notably, in IL-10-deficient mice, sensitization with OVA/Alum+CpG resulted in intense lung neutrophilia and IFN- γ production, indicating that IL-10 is necessary to inhibit subsequent Th1 immunity. Our work highlights the mechanisms of allergy attenuation by CpG and it indicates the potential use of Alum-based formulation with CpG to treat allergic processes.

KEYWORDS

Blomia tropicalis, bronchoalveolar lavage, CpG-ODN, inflammation, lungs

1 | INTRODUCTION

Asthma is a complex respiratory syndrome, which affects around 330 million people worldwide.¹ In Brazil, asthma prevalence occupies the eighth position worldwide with thousands of hospitalizations, high number of deaths, and elevated burden to public health care.² Allergic asthma is essentially dependent on T helper type 2 (Th2) lymphocytes that, thorough cytokines/chemokines, mediate all cardinal features of

asthma such as airway obstruction due to bronchoconstriction, mucus hyper-production along with high levels of IgE.^{3,4}

Exposure to mites found in domestic dust (HDM) is one of the main risk factors for asthma development.⁵ *Blomia tropicalis*, part of the Glycyphagidae family, is one of the most prevalent species of domestic mites in tropical and subtropical countries, including Brazil.⁶ Allergic patients with asthma or rhinitis symptoms show high levels of IgE against *B. tropicalis* Ags.⁷

Murine models have been developed to study asthma mechanisms and ovalbumin (OVA) has been the most widely used allergen. However, a major criticism is the fact that OVA is a food allergen. Therefore, animal models have been adapted to airborne allergens derived from HDM.^{8,9}

Abbreviations: ACA, active cutaneous anaphylaxis; Alum, aluminum gel; ASA, active systemic anaphylaxis; BAL, bronchoalveolar lavage; CpG-ODN, CpG oligodeoxynucleotides; HDM, house dust mite; i.d., intradermal injection; i.n., intranasal or intranasally; ICB IV-USP, Institute of Biomedical Sciences; IL-10^{-/-}, IL-10-deficient; MyD88^{-/-}, MyD88-deficient; PAS, Periodic Acid Schiff.

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Previous work of our group using the OVA-model showed that the absorption of endotoxin LPS to alum adjuvant during the sensitization phase inhibited the development of Th2 responses, including IgE production.¹⁰ However, using *B. tropicalis* as allergen, the adsorption of endotoxin induced a neutrophilic lung inflammation.⁹ The activation of TLRs by endotoxin LPS in the OVA model dampened Th2 immunity without shifting toward Th1/Th17 immunity while in the *B. tropicalis* model it was observed a shift from Th2 toward a Th1/Th17 mediated lung inflammation.⁹ More recently, we have shown that CpG-ODN, a TLR9 agonist, during sensitization to OVA was the most effective TLR agonist in attenuating Th2-mediated allergic responses. This activity was dependent on MyD88 and IL-10 molecules.¹¹ Since CpG-ODN was efficient in dampening Th2 activities and it is an adjuvant approved for use in humans,¹² in the present work, we tested whether the adsorption of CpG during the sensitization to *B. tropicalis* could have a prophylactic effect in preventing *B. tropicalis*-induced experimental asthma.

2 | MATERIAL AND METHODS

2.1 | Mice

Six- to 12-week-old female C57BL/6 WT mice, MyD88-deficient or IL-10-deficient on C57BL/6 background were originally purchased from Jackson Laboratories (Bar Harbor, ME, USA) and bred for many generations at the breeding unit of the Institute of Biomedical Sciences, University of São Paulo (ICB IV-USP). Mice were kept in specific-pathogen free conditions with 5 animals per cage, in an individually ventilated caging system (Alesco, Monte Mor, São Paulo, Brazil). Twelve-hour light/dark cycle, temperature-controlled rooms, food and water ad libitum, cardboard tubes, and towel paper were used for environmental enrichment. All experiments involving laboratory animals were approved by the "Ethics Committee for Animal Use" from the ICB IV-USP, our Institutional Animal Care and Use Committee (Ethical Protocol Number 149/2011). The procedures are according to the Brazilian National Law number 11794 from August 10, 2008, which regulates all research activities involving animal use in the country.

Anesthesia was performed previous to sensitization and challenge procedures, with i.m. preanesthetic use of Acepromazine 5 mg/kg (Rhobifarma, São Paulo, Brazil) and ketamine 10 mg/kg (Rhobifarma, São Paulo, Brazil) plus Xylazine 100 mg/kg (Rhobifarma, São Paulo, Brazil) by the i.p. route. Euthanasia was performed with Halothane inhalation (Cristalia, São Paulo, Brazil) before blood collection, bronchoalveolar lavage (BAL), and lung removal procedures. None of the animals used in the present work became ill or died prior to the experimental endpoint.

2.2 | *Blomia tropicalis* extract

Lyophilized standardized allergen extract of *B. tropicalis* (registration no. 1708) batch 2021/0 was manufactured by BIOCEN (Cuba). The extract was partially purified by ultrafiltration with a 10 kDa cut-

off. Allergenic and protein composition was determined by western blotting IgE and SDS-PAGE. Allergenic activity was measured by in vivo skin prick test in allergic patients by parallel application of 3 dilutions. The activity was expressed in biological units, which is related to the skin reaction size produced by Histamine 10 mg/ml.

2.3 | Alum gel preparation

The aluminum gel (Al(OH)₃) was prepared by precipitation of ammonium aluminum sulfate dodecahydrate (AlH₄(SO₄)₂·12 H₂O, Alfa Aesar, MA) with an excess of 1 N NaOH (Synth, SP, Brazil). Aluminum hydroxide (Alum) was suspended in water (Milli Q, Ontario, Canada), washed 5 times, and centrifuged at 3000 rpm for 15 min. The final precipitate was suspended again in water and the final concentration was determined by calculating 1 ml of dry solution.

2.4 | Sensitization protocol to *B. tropicalis* extract with or without Alum administered by the subcutaneous route

Mice were sensitized with *B. tropicalis* extract (10 µg) or PBS (0.2 ml) by the s.c. route in the nape of the neck of each animal with or without Alum (1.6 mg) on days 0 and 7, and subsequently challenged with *B. tropicalis* (10 µg) or PBS (40 µl) by the intranasal (i.n.) route on days 14 and 21. Analyses were performed 24 h after the last challenge.

2.5 | Sensitization protocol with *B. tropicalis* extract administered by the intranasal or subcutaneous routes for 3 consecutive days

Mice were sensitized with *B. tropicalis* extract (50 µg) or PBS (40 µl) by the intranasal (i.n.) or s.c route on days 0, 1, and 2 and challenged with i.n. with *B. tropicalis* extract (10 µg) or PBS (40 µl) on days 7, 14, and 21. Analyses were performed 24 h after the last challenge.

2.6 | Sensitization protocol with CpG-ODN

Mice were sensitized with the *B. tropicalis* extract (10 µg) by the s.c. route in the nape of the neck of each animal, associated or not to CpG-TLR9 agonist (10 µg) (Invivogen, San Diego, USA) adsorbed to Alum (1.6 mg) on days 0 and 7. Challenges with *B. tropicalis* extract (10 µg) by i.n. route was performed on days 14 and 21. Analyses were performed 24 h after the last challenge.

2.7 | Airway responsiveness

Airway responsiveness to 2 doses of inhaled Methacholine (Sigma-Aldrich, Saint Louis, USA) (12 and 25 mg/ml, respectively) was determined using a single-chamber, whole-body plethysmograph (Buxco Electronics Inc., Wilmington, NC, USA). After each nebulization with Methacholine, recordings were taken for 5 min. The Penh values measured during each of the 5-min sequence were averaged and expressed

for each methacholine concentration. Results represent the area under the curve obtained with increasing doses of methacholine.

2.8 | BAL fluid and blood collection

Blood samples were collected by cardiac puncture, centrifuged, and serum was stored at -20°C . The BAL was obtained after lung washing with 1 ml of cold PBS via the trachea. Total and differential cell counts of BAL fluids were determined by hemacytometer (Sigam-Aldrich, Saint Louis, USA) and cytospin (Thermo Fisher Scientific, Waltham, USA) preparation, stained with H&E (Instant-Prov, Newprov, SP, Brazil).

2.9 | Histopathological analysis

After BAL collection, lungs were perfused with 10 ml of cold PBS through the right ventricle, fixed in 10% PBS-formalin for 24 h, and then in 70% ethanol until embedding in paraffin. The lung tissue from caudal lobule was cut to a thickness of 5 μm and stained with H&E for visualization of the cell infiltrate or with Schiff Periodic Acid (PAS) for evaluation of production of mucus. Lung inflammation was scored by measuring the area of peribronchial infiltrate (μm^2), per length of bronchial basal membrane (μm). Mucus score was calculated by measuring the area of mucus formation in bronchioli (μm^2) per bronchial basement membrane (μm) or bronchial area.

2.10 | ELISA for total IgE and cytokine determinations

Total mouse IgE was determined by sandwich-ELISA kit, BD OptEIA ELISA Set (BD Bioscience, San Diego, USA), according to the manufacturer's recommendation. Values were expressed in $\mu\text{g/ml}$, deduced from a standard curve of recombinant Ab (100 ng/ml) ran in parallel. The samples were diluted (1:100) and the limits of detection ranged from 1.6 to 100 ng/ml. IL-5, IFN- γ , and IL-10 levels were assayed by sandwich-ELISA kit, according to the manufacturer's recommendation BD OptEIA ELISA Set (BD Bioscience, San Diego, USA). Values were expressed in pg/ml, deduced from a standard curve of recombinant cytokines (1000, 4000, and 2000 pg/mL, respectively) ran in parallel. The limits of detection ranged in IL-5 from 15.6 to 1000 pg/mL, IFN- γ from 62.5 to 4000 pg/mL, and IL-10 from 31.3 to 2000 pg/mL.

2.11 | Active systemic anaphylaxis temperature assay

C57BL/6 mice were sensitized to *B. tropicalis* (*B. tropicalis*/Alum) or to *B. tropicalis*+CpG (*B. tropicalis* + CpG/Alum) on days 0 and 7 and at day 14 challenged with i.n. *B. tropicalis* extract. At day 21, mice were challenged by i.v. route with 20 μg of *B. tropicalis* extract and systemic anaphylaxis assay was determined by body temperature drop. An electronic rectal TC-2000 thermocouple meter thermometer (Sable Systems, Nevada, USA) was used to measure the body temperature of animals at different time periods (0, 15, 30, 60, 90, and 180 min). Temperature

delta was determined by the difference between final temperature and initial temperature.

2.12 | Active cutaneous anaphylaxis assay

C57BL/6 mice were sensitized to *B. tropicalis* extract and challenged as described above. At day 21, the animals received an intradermal injection (i.d.) on the back containing *B. tropicalis* extract (20 μg) or PBS, followed by an i.v. injection of Evans Blue dye (1 mg/ml) to determine active cutaneous anaphylaxis. After 30 min, the animals were euthanized and skin of the dorsal region was removed for visualization of the reaction. For quantification, the 2 skin spots (PBS and *B. tropicalis* extract) were removed, weighed, and the dye extraction was performed with formamide for 24 h. The skin was withdrawn and 100 μl of the extracted contents present in each test tube was pipetted into a 96-well plate. The reading was given at wavelength of 610 nm and the results expressed as $\mu\text{g/ml}$, deduced from a standard curve with known concentrations of Evans blue dye.

2.13 | Flow cytometric analysis

Cells were isolated from the lungs as previously described.¹³ Briefly, the lungs were perfused with cold PBS, tissue was collected, chopped with scissors and digested with RPMI-1640 medium (Sigma, St Louis, MO) containing 1 mg/ml Collagenase IV (Sigma-Aldrich) and 0.5 mg/ml DNase I (Sigma-Aldrich), by continuous stirring at 37°C for 25 min. Digested tissue was immediately diluted in complete RPMI-1640 medium containing: 10% FCS, 100 $\mu\text{g/ml}$ Penn/Strep, 25 mM HEPES, 2 mM L-glutamine, and 50 mM μ -mercaptoethanol, and mashed through 70 μm cell strainers (BD Biosciences). Cell suspensions were spun down and resuspended in complete RPMI-1640 medium. Draining lymph nodes from head-neck region (cervical, axillary, and brachial) from naïve or *B. tropicalis*-sensitized mice were collected at day 14, dissected, passed through a cell strainer mash, counted, and resuspended in complete RPMI-1640 medium. Cell counting was performed in a Neubauer hemocytometer with Trypan blue, to evaluate cell viability. Approximately $1-2 \times 10^6$ cells per well were transferred to a 96-well plate. Cells were stimulated with a solution containing 50 ng/ml PMA (Sigma, USA), 500 ng/ml Ionomycin (Sigma, USA), and Golgi-plug (BD Biosciences) and then incubated for 4 h, at 37°C and 5% CO_2 . After stimulation, the cells were washed and incubated with a fixable viability probe (Live/Dead, Life technologies) for ~ 20 min, thus enabling the cell viability analysis in the flow cytometer. Subsequently, after washing, the cells were fixed and permeabilized using the CitoFix/CitoPerm permeabilization kit (BD Biosciences) for 20 min. After the fixation and permeabilization phase, the cells were then incubated for ~ 1 h with the Ab mix containing the following antibodies: anti-CD3 (BD-Biosciences, clone 17A2), anti-CD4 (BD-Biosciences, clone GK1.5), anti-CD44 (BD-Biosciences, clone IM7), anti-IL4 (BD-Biosciences, clone 11B11), anti-IL-5 (BD-Biosciences, clone TRFK5), anti IFN- γ (BD-Biosciences, clone XMG1.2), and anti-Foxp3 (BD-Biosciences, clone FJK-16S). Cell acquisition was performed on a BD LSRFortessa X20 cell analyzer (BD Biosciences) using FACSDiva

software (BD Biosciences) and data were analyzed using FlowJo software (TreeStar Inc.).

2.14 | Statistical analyses

Statistical analyses were performed using GraphpadPrism (V.5; Graph-Pad Software, USA). Differences were considered statistically significant when P -value ≤ 0.05 . Data represent the mean $\pm$ SE.

3 | RESULTS

3.1 | Protocols of allergic sensitization to HDM *B. tropicalis* and characterization of (*B. tropicalis*) experimental asthma model

In order to ascertain the best protocol of allergic sensitization to *B. tropicalis* extract, we examined 2 routes of sensitization, s.c. versus i.n., and the effect of alum as a Th2 adjuvant on allergic sensitization (Fig. 1A and B). Mice were sensitized with 10 μ g of *Blomia tropicalis* extract administered by s.c. route adsorbed or not to alum adjuvant at days 0 and 7 (Fig. 1A) or sensitized i.n. or s.c. with 50 μ g of *B. tropicalis* extract for 3 consecutive days (0, 1, and 2; Fig. 1B). All groups of animals depicted in Fig. 1A were challenged i.n. with 10 μ g of *B. tropicalis* extract at days 14 and 21 and those in Fig. 1B were challenged i.n. with 10 μ g of *B. tropicalis* extract at days 7, 14, and 21. The magnitude of lung allergic inflammation and the levels of IgE were determined 24 h after last challenge. We found that the total number of cells in BAL fluid increased significantly in mice sensitized with *B. tropicalis* plus alum by s.c. or with i.n. routes, when compared to non-manipulated naïve mice (Fig. 1C). All *B. tropicalis*-treated groups showed increased number of neutrophils and eosinophils in BAL when compared to the naïve (non-manipulated) group (Fig. 1C), but the increase in eosinophils was significantly higher in the *B. tropicalis*/Alum group sensitized by s.c. route than in the other groups (Fig. 1C). Importantly, the serum levels of total IgE were also higher in the *B. tropicalis*/Alum group (Fig. 1D). To further characterize the *B. tropicalis* model of allergic asthma and to compare i.n. versus s.c. sensitization, mice were sensitized by i.n. or s.c. routes (with or without alum) at days 0 and 7, and challenged at days 14 and 21 (Fig. 2A). One day after the first i.n. *B. tropicalis* challenge, lung function was studied by measuring airway responses to increasing doses of methacholine. We found that the airway hyperreactivity to methacholine was significantly increased in all *B. tropicalis*-sensitized when compared to naïve (non-manipulated) mice, as revealed by measuring Penh values and the area under the curve (Fig. 2B and C). Lung mucus formation was determined 1 day after the second *B. tropicalis* challenge, by PAS-stained lung tissue sections. We found that all *B. tropicalis*-sensitized groups showed increased mucus production when compared to naïve mice, without significant difference between *B. tropicalis* groups (Fig. 2C and D). Regarding IgE production, only mice sensitized with alum adjuvant showed a significant increase in the serum concentrations of total IgE (Fig. 2E). We concluded, based on the total number of cells and eosinophils in BAL or in the serum levels of IgE, that sensitization to *B. tropicalis* with

alum adjuvant was the most effective in inducing allergic responses (Figs. 1 and 2). Therefore, we used the s.c. sensitization protocol (*B. tropicalis*/Alum) as a standard protocol for *B. tropicalis*-induced experimental asthma.

3.2 | Sensitization to *B. tropicalis* extract is essential for airway eosinophilic inflammation but not for neutrophil influx

We further investigated whether the airway influx of neutrophils was induced by the activation of innate immunity due to the endotoxin contamination of *B. tropicalis* extract or dependent on the adaptive Th17 immunity due to the sensitization to *B. tropicalis* allergens. For this, we compared the airway inflammation of animals challenged and sensitized to *B. tropicalis* with *B. tropicalis*-sensitized or *B. tropicalis*-challenged animals. Animals received PBS or *B. tropicalis*/Alum on days 0 and 7 and were *B. tropicalis* or PBS challenged at days 14 and 21 (Fig. 3A). *B. tropicalis*-sensitized and *B. tropicalis*-challenged mice showed an increase in total cells in BAL when compared to all other groups (Fig. 2B). Notably, in *B. tropicalis*-challenged mice, the number of neutrophils was similar in sensitized or non-sensitized mice, while only sensitized mice showed an increase of eosinophils in BAL (Fig. 3C). Total and differential cell counts of *B. tropicalis*-sensitized but not *B. tropicalis*-challenged mice were similar to the control group (Fig. 3B and C). These results indicate that eosinophil, but not neutrophil influx, is due to adaptive immunity to *B. tropicalis* allergens while the neutrophil influx is probably due to innate inflammatory response induced by the *B. tropicalis* extract. Notably, a significant increase in IgE production only occurred in *B. tropicalis*-sensitized and *B. tropicalis*-challenged mice (Fig. 3D). Since the *B. tropicalis* extract induced neutrophilic airway inflammation probably via innate immunity, we investigated the role of MyD88 adaptor molecule in our *B. tropicalis* asthma model. For this, we compared the responses of MyD88-deficient (MyD88^{-/-}) mice with those obtained in WT C57BL/6 mice. Mice were sensitized on days 0 and 7 and challenged days 14 and 21 with *B. tropicalis* extract, as described before. BAL cell counts (total and differential) and IgE levels were determined. We found that MyD88^{-/-} mice did not develop airway allergic inflammation as revealed by total and differential cell counts in BAL or IgE production when compared to WT B6 mice (Supplementary Fig. 1A–C). These results indicate that MyD88 expression is crucial for the induction of asthma-like responses by the *B. tropicalis* extract.

3.3 | Sensitization with Alum plus CpG prevents the development of *B. tropicalis*-induced allergic responses

Having established the best protocol to induce asthma-like response to *B. tropicalis* extract, we then investigated the effect of Alum-based CpG formulation on the development of allergic responses. For this, mice were sensitized with *B. tropicalis* extract/Alum adsorbed or not with CpG (10 μ g) and then i.n. challenged with *B. tropicalis* as depicted in Fig. 4A. As expected, animals sensitized and challenged with *B. tropicalis* extract showed an increase in the total number of cells

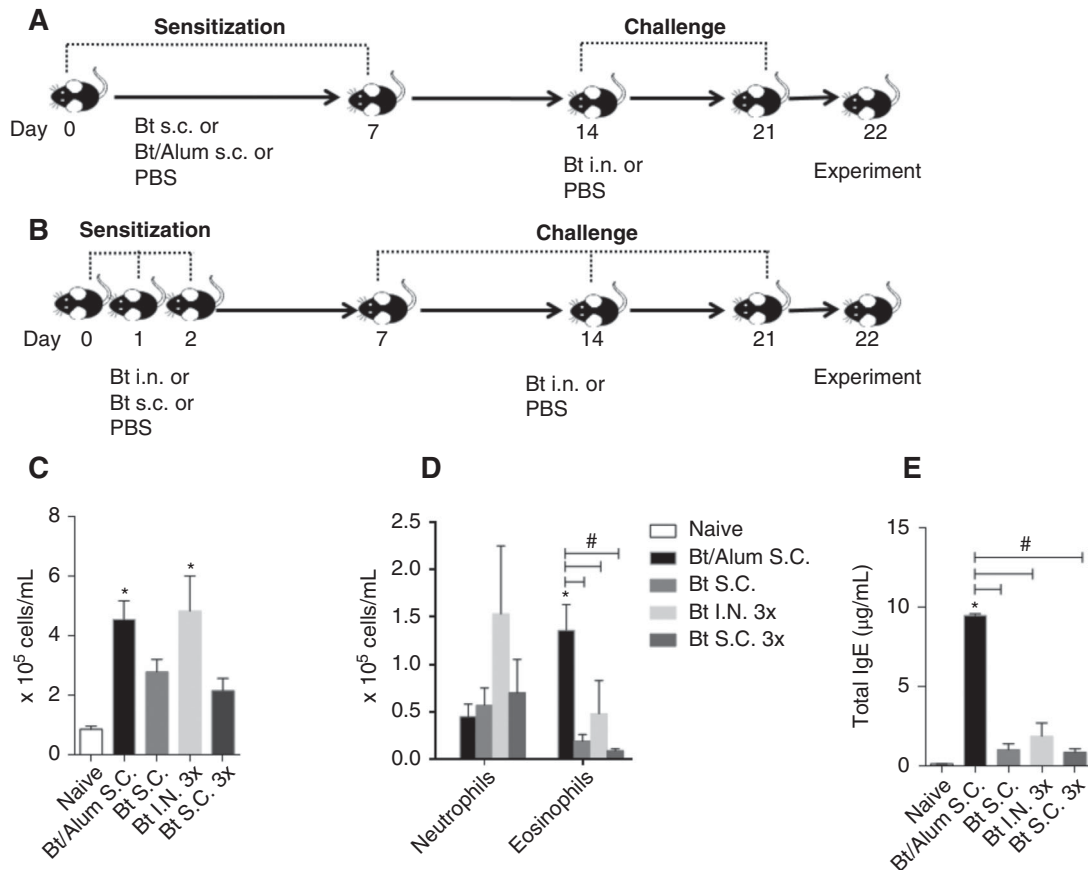


FIGURE 1 Comparison of different protocols of allergic sensitization to HDM *Blomia tropicalis*. WT C57BL/6 mice were sensitized (A) via s.c. with *Blomia tropicalis* extract or with *Blomia tropicalis* adsorbed to alum (*Blomia tropicalis*/Alum) on days 0 and 7 and challenged on days 14 and 21 or (B) with i.n. or s.c. Bt on days 0, 1, and 2 and challenged with *Blomia tropicalis* on days 7, 14, and 21. Samples were obtained on day 22. (C) Total cell and (D) differential cell counts in BAL; (E) Total IgE serum levels. Naive group consisted of non-manipulated animals. Results are expressed as mean $\pm$ SE of five mice per group and are representative of two experiments. One-way ANOVA * = $p < 0.05$ different from Naive group; # different between groups

in BAL as well as in neutrophils and eosinophils when compared to the naïve group (Fig. 4B and C), while in animals sensitized with *B. tropicalis*+CpG/Alum, the total number of cells and eosinophils, but not neutrophils, were significantly lower when compared with animals sensitized with *B. tropicalis*/Alum (Fig. 4B and C). Accordingly, airway hyperreactivity to increasing doses of methacholine and total IgE concentrations were also lower in the *B. tropicalis*+CpG/Alum group than in the *B. tropicalis*/Alum group (Fig. 4D-E). Histopathological examination of lung sections revealed that lung cellular infiltrates (Fig. 4F and G) and mucus formation (Fig. 4F and H) were significantly lower in the *B. tropicalis*+CpG/Alum group than in the *B. tropicalis*/Alum, indicating that CpG significantly inhibited lung inflammatory cell infiltration and mucus formation (Fig. 4G and H). Flow cytometry analysis of IL-5 or IFN γ -producing CD3⁺CD4⁺ T cells present in the lung tissue showed that the frequency of IL-5-producing T cells were increased in the *B. tropicalis*/Alum group when compared to naïve mice and this increase was inhibited in the *B. tropicalis*+CpG/Alum group. Differently, IFN γ -producing T cells were almost absent in all groups (Fig. 5A–C). Cytokine content in BAL showed a decrease of IL-5 in the *B. tropicalis*+CpG/Alum group when compared to the *B. tropicalis*/Alum and no increase of IFN γ was observed (Fig. 5D and E), confirming the data obtained by flow

cytometry analysis. Importantly, we found that IL-10 levels in BAL were higher in the *B. tropicalis*+CpG/Alum group than in the other groups (Fig. 5F). These results clearly indicate that CpG dampens Th2 responses without inducing immune deviation toward Th1 cells, while IL-10 production in the lung might be involved in the inhibition of Th2 responses. We could not determine the cell type responsible for IL-10 production (myeloid or lymphoid) because we did not obtain a positive stain to intracellular IL-10 due to an intrinsic technical problem. All in all, sensitization to *B. tropicalis* in the presence of CpG prevented the development of key features related to allergic asthma pathology such as eosinophilic influx and IL-5 production, airway hyperreactivity, mucus formation, peribronchial inflammation, and elevated levels of IgE, without shifting the pulmonary response to a Th1 pattern.

3.4 | IL-10 is dispensable for CpG-induced inhibition of IL-4-producing Th2 cells in draining lymph nodes

Since WT C57BL/6 mice sensitized with *B. tropicalis*+CpG/Alum presented decreased airway allergic responses and increased IL-10 production, we decided to investigate the role of IL-10 on Th2 sensitization. For this, we compared the frequency and the absolute

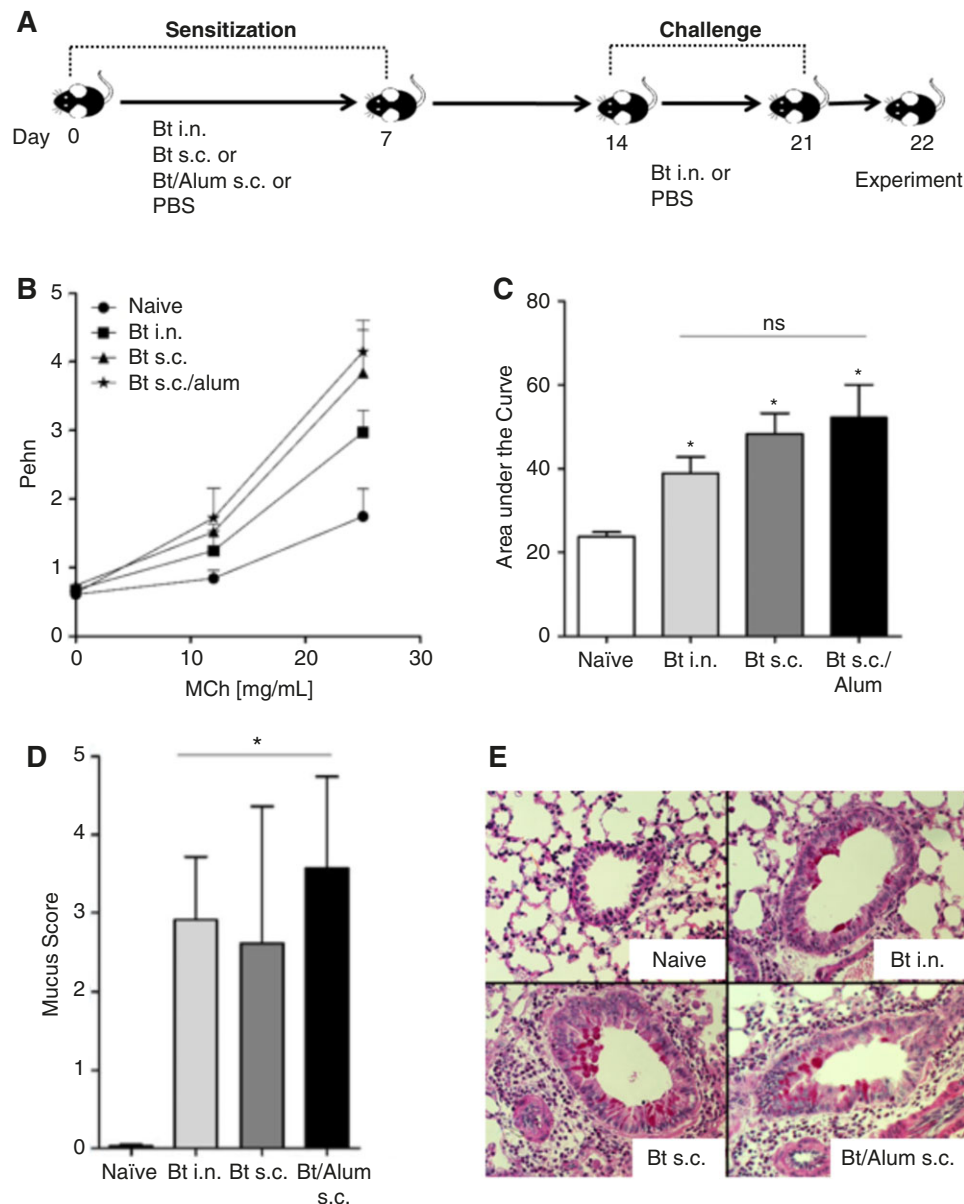


FIGURE 2 Characterization of HDM *Blomia tropicalis* experimental asthma model. (A) WT C57BL/6 mice were sensitized i.n. or s.c. with *Blomia tropicalis* extract (Bt) or s.c. with Bt adsorbed to alum (Bt/Alum) on days 0 and 7 and challenged on days 14 and 21. (B) Airway responsiveness (Penh values) to increasing doses of inhaled methacholine in conscious unrestrained mice were determined using whole-body plethysmography and expressed as (C) area under the curve (AUC); (D) Mucus score determined as area/basement membrane and (E) Representative microphotographs of lung sections PAS-stained. Naive group consisted of non-manipulated animals. Results are expressed as mean $\pm$ SE of 5 mice per group. One-way ANOVA * = $p < 0.05$ different from Naive group

number of activated T helper cells (CD4+CD44+) in draining lymph nodes of WT and IL-10^{-/-} mice sensitized with *B. tropicalis*/Alum or *B. tropicalis*+CpG/Alum. We found that both C57BL/6 WT and IL-10^{-/-} mice sensitized with *B. tropicalis*/Alum showed a significant increase in the frequency and in the absolute number of IL-4-producing CD4+CD44+ T cells when compared to control mice (Fig. 6A and C left panel). In contrast, sensitization with *B. tropicalis*+CpG/Alum resulted in a significant decrease in the frequency and in the absolute number of IL-4-producing CD4+ T cells in C57BL/6 WT or IL-10^{-/-} animals when compared to the *B. tropicalis*/Alum group (Fig. 6A and

C left panel). It indicates that the inhibition of Th2 sensitization does not require IL-10. Notably, the frequency and the absolute number of IFN- γ -producing CD4⁺ Th1 cells were not significantly different in WT or in IL-10^{-/-} mice (Fig. 6A and C middle panel). Moreover, no significant changes in the frequencies or absolute numbers of Foxp3+ Tregs cells were observed between *B. tropicalis*/CpG and *B. tropicalis*+CpG/Alum groups in both C57BL/6 WT and IL-10-KO mice (Fig. 6B and C right panel). Taken together these results suggest that addition of CpG to *B. tropicalis*/Alum impairs Th2 sensitization without concomitant increase of Th1 or Treg cells.

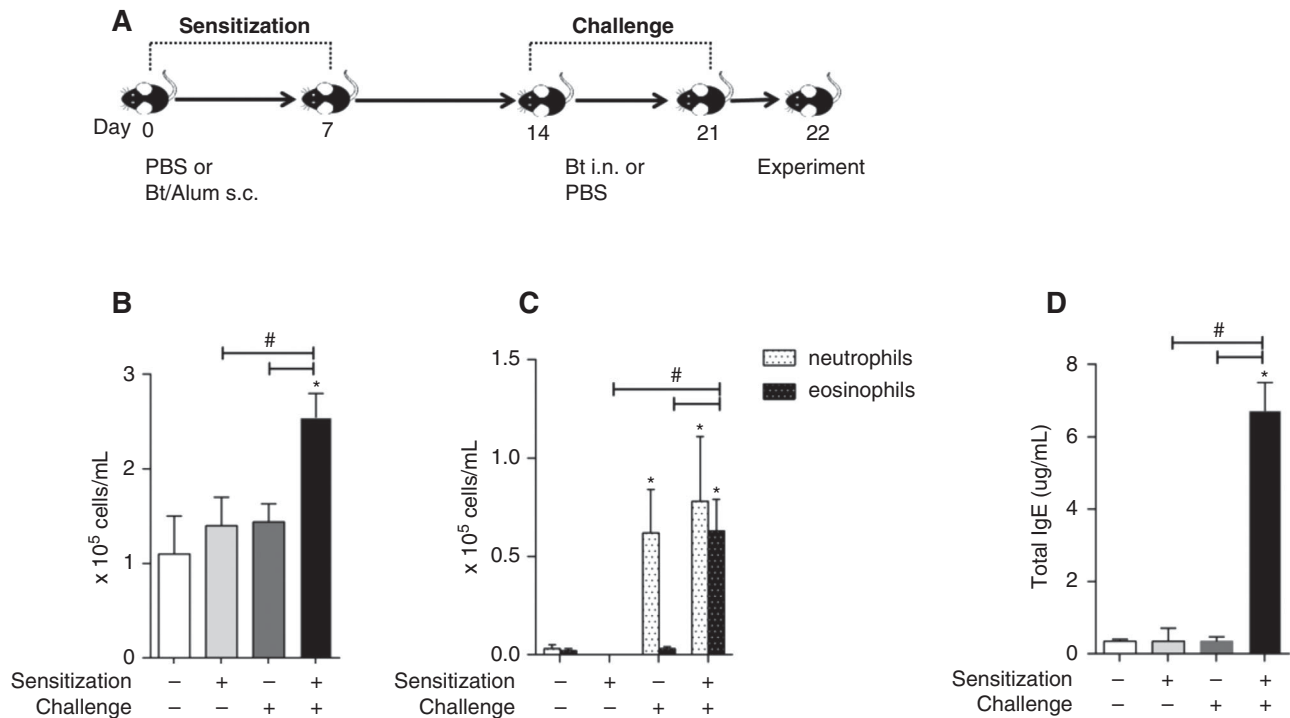


FIGURE 3 Sensitization to *Blomia tropicalis* extract is essential for airway eosinophilic inflammation but not for neutrophil influx. C57BL/6 WT mice were sensitized with Bt/Alum or PBS on days 0 and 7 and challenged with Bt on days 14 and 21. Samples were obtained on day 22. (A) Experimental protocol. (B) Total cell and (C) differential cell counts in the BAL; (D) Total IgE. Naive group consisted of non-manipulated animals. Results are expressed as mean $\pm$ SE of 3–5 mice per group and are representative of 2 experiments. One-way ANOVA * P < 0.05 different from Naive group; # different between groups; ns, not significant

3.5 | IL-10 is fundamental to preclude development Th1 immunity after sensitization with *B. tropicalis*+CpG/Alum

We have previously shown using the OVA-asthma model that IL-10-deficient mice developed an intense Th1-like airway inflammation.¹¹ Therefore, we tested our protocols of *B. tropicalis* sensitizations and challenge in IL-10^{-/-} mice. *B. tropicalis*/Alum sensitized IL-10^{-/-} mice developed airway inflammation with infiltration of neutrophils and eosinophils as revealed by total and differential cell counts (Fig. 7A and B). However the increased infiltration of eosinophils was not accompanied by an increase of IL-5 production in BAL (Fig. 7C). In addition, *B. tropicalis*/Alum group showed higher levels of IgE than the Naïve or *B. tropicalis*+CpG groups (Fig. 7D). Strikingly, IL-10^{-/-} animals sensitized with *B. tropicalis*+CpG/Alum, instead of showing a decreased airway inflammation as shown before in C57BL/6 WT mice, showed a significantly increased influx in the total number of inflammatory cells in BAL when compared to the *B. tropicalis*/Alum group (Fig. 7A) that was dominated by a remarkable increase of neutrophil in BAL (Fig. 7B). Although the absolute number of eosinophils was not decreased in IL-10^{-/-} mice sensitized with *B. tropicalis*+CpG/Alum when compared to the *B. tropicalis*/Alum group (Fig. 7B right panel), the percentage of eosinophils in this group was roughly 50% lower than in the *B. tropicalis*/Alum group (Fig. 7B left panel). In IL-10^{-/-} mice, we could not detect IL-5 production in the BAL of any group. In contrast, the *B. tropicalis*+CpG/Alum group presented high levels of IFN- γ (Fig. 7C) and a significant decrease

in the levels of IgE when compared to the *B. tropicalis*/Alum group (Fig. 7D). These results indicate that in IL-10^{-/-} mice, the addition of CpG to *B. tropicalis*/Alum result in the development of intense airway neutrophilic inflammation associated with increased production of IFN- γ , suggesting a dominance of Th1 immunity. Histopathological analysis of H&E stained lung sections confirmed that the inflammation was more intense in the *B. tropicalis*+CpG/Alum than in the *B. tropicalis*/Alum group (Fig. 7E and G). PAS stained sections showed a decreased mucus production in the *B. tropicalis*+CpG/Alum when compared to the *B. tropicalis*/Alum group, reinforcing the inhibition of Th2 immunity by CpG (Fig. 7F and H). We concluded that in the absence of IL-10, *B. tropicalis*+CpG/Alum sensitization prevented Th2 immunity and induced Th1 pattern of lung immunity with a dominant influx of neutrophils.

3.6 | CpG impairs the development of *B. tropicalis*-specific active cutaneous or systemic anaphylaxis

Since sensitization with *B. tropicalis*+CpG/Alum impaired the production of IgE Abs when compared to *B. tropicalis*/Alum sensitization, we tested more specifically whether CpG could impair anaphylaxis in vivo mediated by anaphylactic Abs. To test this, we determined the magnitude of active cutaneous anaphylaxis measured in vivo by Evans blue extravasation¹⁴ upon intradermal injection of *B. tropicalis* extract or systemic anaphylaxis monitored by drop in body temperature¹⁵ upon

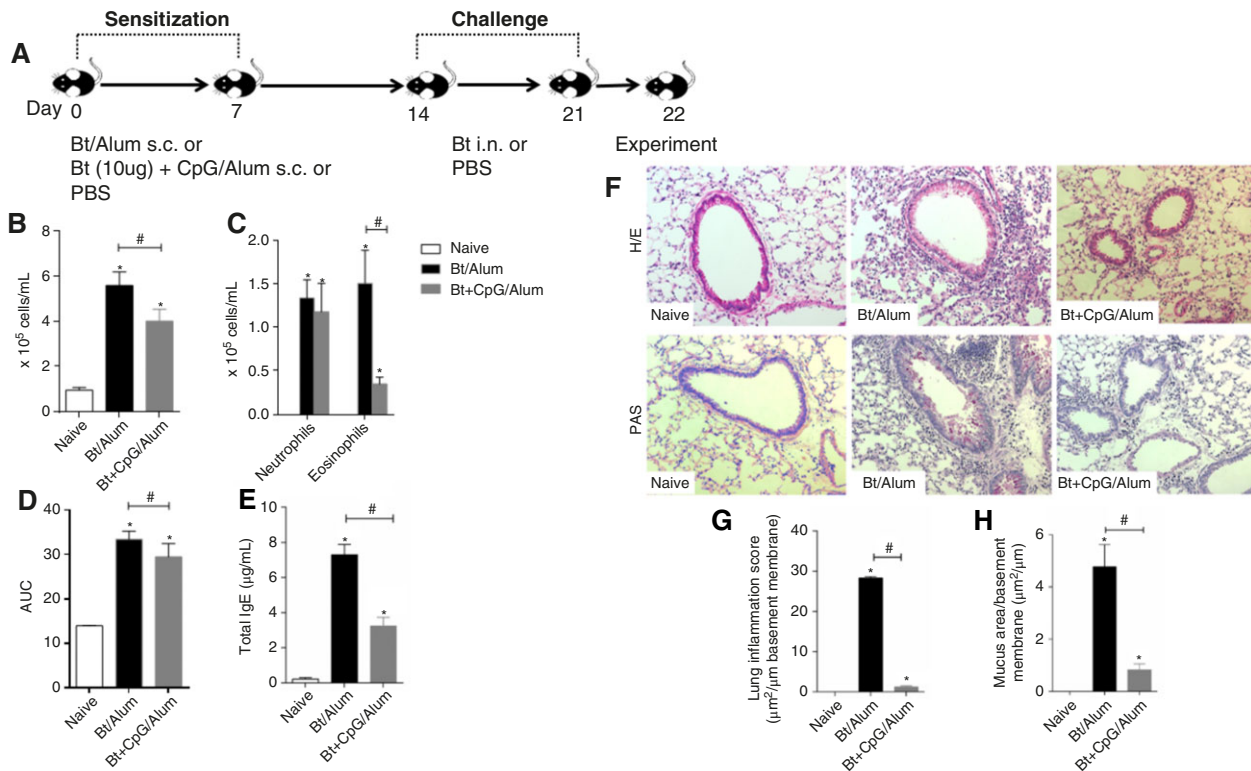


FIGURE 4 Sensitization with Alum plus CpG containing formulation prevents the development of *Blomia tropicalis*-induced allergic responses. C57BL/6 WT mice were sensitized with *Blomia tropicalis*/Alum or with *Blomia tropicalis*+CpG/Alum on days 0 and 7 and challenged i.n. with *Blomia tropicalis* on days 14 and 21. Samples were obtained on day 22. (A) Experimental protocol, (B) total cell, and (C) differential cell counts in the BAL; (D) total IgE, (E) area under the curve (AUC) to increasing doses of methacholine. (F) Representative microphotographs of lung sections stained with HE and (G) PAS, (H) lung inflammation score, and (I) mucus area/basement membrane score. Naive group consisted of non-manipulated animals. Results are expressed as mean $\pm$ SE of 3–5 mice per group and are representative of 3 experiments. One-way ANOVA * $P < 0.05$ different from Naive group; # $P < 0.05$ different from *Blomia tropicalis*/Alum group

i.v. *B. tropicalis* administration. We found that *B. tropicalis*/Alum sensitized mice developed an intense dye extravasation upon intradermal *B. tropicalis* injection when compared to the naïve group that showed a very discrete dye extravasation (Fig. 8A and B). Importantly, animals sensitized with *B. tropicalis*+CpG/Alum also showed a discrete dye extravasation (Fig. 8C), which was significantly lower when compared to the allergic *B. tropicalis*/Alum group, as revealed by ACA score (Fig. 8D). In line with these results, analysis of systemic anaphylaxis also showed that mice from the *B. tropicalis*/Alum group developed a drop in the body temperature upon i.v. *B. tropicalis* administration while this was not observed in *B. tropicalis*+CpG/Alum sensitized or naïve groups (Fig. 8E). These results indicate that sensitization in the presence of CpG prevents *B. tropicalis*-specific active cutaneous or systemic anaphylaxis.

4 | DISCUSSION

In the present work, we determined the best sensitization protocol to induce asthma-like responses using the mite extract from *B. tropicalis*. In order to mimic natural sites of airborne allergen sensitization, we used two different routes to sensitize B6 mice to *B. tropicalis* allergens, the subcutaneous route mimicking a percutaneous sensitization and the intranasal route mimicking airway exposure. Although all

protocols tested for *B. tropicalis* sensitization resulted in increased influx of inflammatory cells in the airways upon *B. tropicalis* challenge, only s.c. sensitization to *B. tropicalis* in the presence of alum adjuvant resulted in high levels of IgE production, a cardinal feature of allergic diseases. Therefore, we used this protocol to perform all subsequent experiments. We found that MyD88 adaptor molecule, which mediates signaling downstream of IL-1R and TLRs,¹⁶ was fundamental for the induction of allergic airway inflammation and IgE production. This is in contrast to what we have previously found with the OVA-model of asthma, in which the MyD88 molecule was dispensable for allergic inflammation and IgE production.¹¹ However, it should be mentioned that other studies using OVA-models found a crucial role for MyD88 and activation NLRP3 inflammasome in the development of allergic airway inflammation, both in alum adjuvant-dependent and in an adjuvant-free OVA model of allergic asthma.^{17,18} The controversy around the requirement of MyD88 for allergic sensitization in the OVA model remains unsettled, but could be related to differences in protocols of sensitization or preparations of alum adjuvants used. Nevertheless, our results with *B. tropicalis* are in line with a work showing that MyD88 molecule is essential for HDM sensitization.¹⁹

Although the use alum for experimental models of allergy sensitization does not represent the real scenario of human allergic sensitizations, its use provides a robust and reproducible model of allergic

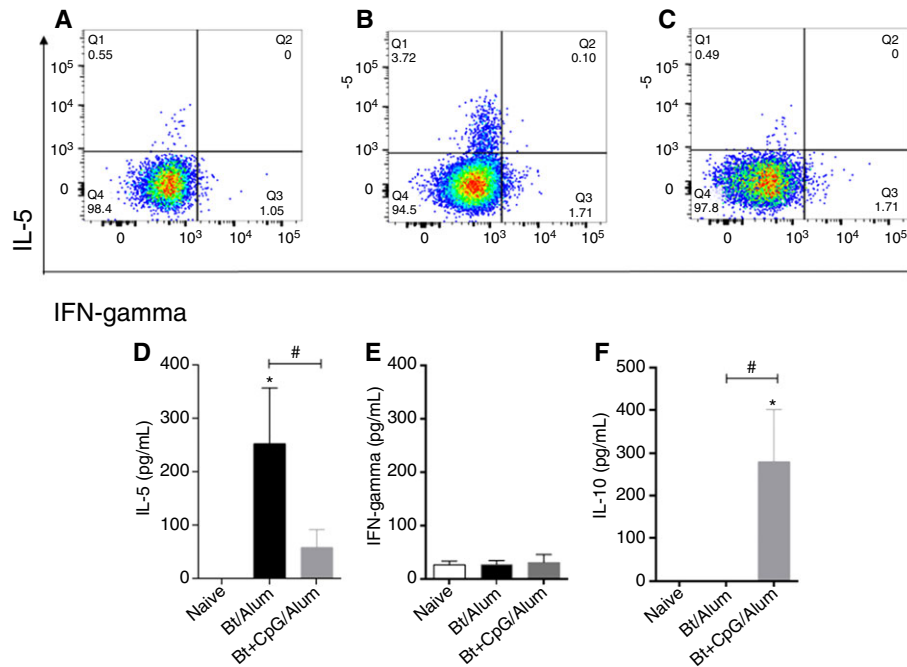


FIGURE 5 Cytokine production. C57BL/6 WT mice were sensitized with *Blomia tropicalis*/Alum or with *Blomia tropicalis*+CpG/Alum on days 0 and 7 and challenged with i.n. *Blomia tropicalis* on days 14 and 21. Samples were obtained on day 22. FACS analysis of representative panel for IL-5 and IFN- γ staining in CD3⁺CD4⁺CD45⁺ and Live/Dead-cells. (A) Naïve group, (B) *Blomia tropicalis*/Alum and (C) *Blomia tropicalis*+CpG/Alum groups; of lung homogenates (3–5 animals/group). (D) IL-5, (E) IFN- γ , and (F) IL-10 levels in the BAL. Naïve group consisted of non-manipulated animals. Results are expressed as mean $\pm$ SE of 3–5 mice per group and are representative of 3 experiments. One-way ANOVA * P < 0.05 different from Naïve group; # P < 0.05 different from *Blomia tropicalis*/Alum group

diseases.²⁰ For instance, here we showed that animals sensitized with *B. tropicalis* extract adsorbed onto alum develop airway eosinophilic inflammation, mucus production, peribronchial cellular infiltrates, lung infiltrating CD4⁺ T cells producing IL-5, airway hyperresponsiveness and high levels of IgE, confirming previous works.⁹ Notably, we found that the addition of CpG to *B. tropicalis*/Alum sensitization attenuated significantly the development of all Th2-associated allergic responses in WT B6 mice. Importantly, the inhibition of Th2 pathology by CpG was not associated with a shift of the pulmonary response toward a Th1 pattern since there was no increase in the IFN- γ -producing T cells in the lung. Accordingly, the histological scores of lung inflammation showed a decrease mucus formation, a Th2 associated parameter, and a significant decrease in lung cellular infiltrates in the *B. tropicalis*+CpG/Alum when compared to the *B. tropicalis*/Alum group. This indicates that addition of CpG during sensitization results in a nonpolarized lung T cell immunity. Since the inhibition of lung Th2 immunity by CpG was associated with increased production of IL-10 in the BAL, we phenotyped CD4⁺ T helper cells subsets present in the draining lymph nodes of IL-10^{-/-} and compare with WT mice, sensitized with *B. tropicalis* extract/Alum or *B. tropicalis*+CpG/alum before i.n. *B. tropicalis* challenge. We found that *B. tropicalis*/Alum sensitization increased the frequency and the absolute number of Th2 cells in both strains of mouse, being more evident in WT than in IL-10^{-/-} mice, reinforcing the pro-Th2 activity of IL-10.²¹ In contrast, in *B. tropicalis*+CpG/Alum-sensitized mice, the Th2 increase was not observed in WT or in IL-10^{-/-} mice, indicating that IL-10 is dispensable

for CpG-mediated inhibition of Th2 development. It should be noted that although CpG is known to increase Th1²² or Treg activities,^{23,24} in our model, we could not detect an increase of Th1 or Treg cells. It is possible that during the *B. tropicalis* sensitization, dendritic cells receive a negative signal from endocytosed CpG that precludes the necessary signals required for effective Th2 sensitization.

A completely different scenario emerged when we analyzed lung immunity in IL-10-deficient mice challenged with *B. tropicalis*. In this situation, *B. tropicalis* extract/Alum group developed Th2 immunity, but IL-5 levels were not detected. Although Th2 immunity was inhibited in *B. tropicalis*+CpG/Alum (total IgE, mucus production, and percentage of eosinophils but not the total number of eosinophils) as opposed to the *B. tropicalis*/Alum group, a concomitant development of intense neutrophilic airway inflammation associated with very high levels of IFN- γ in BAL was detected, indicating that the main effect of IL-10 is to hold up the development of ensuing lung Th1 immunity. These results are in line with our previous work using the OVA-model of asthma.¹¹ Accordingly, a similar approach has been used in tumor immunology where CpG plus anti-IL-10 receptor treatment showed robust antitumor activity, higher than CpG or anti-IL-10R alone.²⁵ It is puzzling to understand why augmented Th1 immunity could not be detected in IL-10^{-/-} mice draining lymph nodes before *B. tropicalis* challenge, since that it became evident in the lung after the allergen challenge.

Finally, to evaluate the effect of CpG in the in vivo production of anaphylactic Abs, we performed an active cutaneous anaphylaxis, determined by dye extravasation upon intradermal *B. tropicalis*

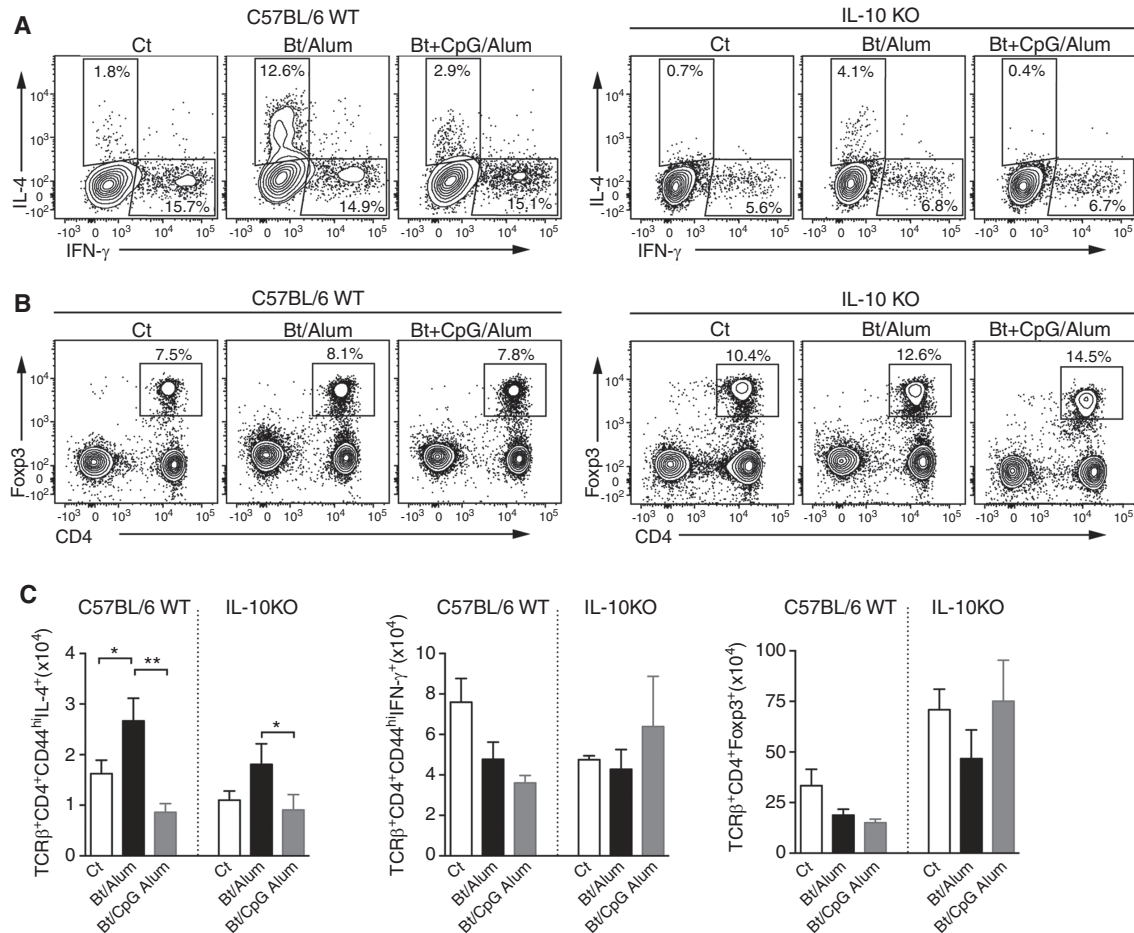


FIGURE 6 Characterization of T helper cell subsets from draining lymph nodes of C57BL/6 WT and IL-10KO mice sensitized with *Blomia tropicalis*/Alum or *Blomia tropicalis*/CpG Alum. C57BL/6 WT ($n = 5$) and IL-10^{-/-} ($n = 4$) mice were sensitized s.c. on days 0 and 7 with *Blomia tropicalis*/Alum or *Blomia tropicalis*+CpG/Alum 1 week later, draining lymph nodes from the head neck region were collected and isolated cells were stimulated with PMA and ionomycin in the presence of Brefeldin. Stimulated cells were stained for intracellular cytokine production and analyzed by flow cytometry. IL-4 and IFN- γ production was analyzed in the population of TCR β^+ CD4⁺CD44^{high} cells that were previously gated on live CD45⁺ cells. (A) Representative dot plots of IL-4 and IFN- γ staining in samples from C57BL/6 WT (left panel) or IL-10 KO mice (right panel). Non-stimulated cells from draining lymph nodes of sensitized or naive mice were stained for intra-nuclear expression of Foxp3 that was analyzed in the TCR β^+ CD4⁺ cells that were previously gated on live CD45⁺ cells. (B) Representative dot plots of Foxp3 expression by CD4⁺ T cells. (C) Absolute numbers of IL-4 (left panel) and IFN- γ (middle panel) producing T cells and Foxp3⁺ CD4⁺ T cells (left panel), data are expressed as the mean $\pm$ SEM obtained in 1 experiment. * $P < 0.05$; ** $P < 0.005$

inoculation, as well as a systemic anaphylaxis, revealed by temperature drop after i.v. *B. tropicalis* challenge. In both assays, the *B. tropicalis*+CpG/Alum group showed a decreased response when compared to the *B. tropicalis*/Alum group, indicating that CpG prevents active anaphylactic reactions. Since in WT B6 mice, the sensitization with *B. tropicalis* extract+CpG/Alum does not result in any overt type of airway inflammation and it blocked *B. tropicalis*-specific anaphylactic Abs, we concluded that this type of sensitization prevents the alum's Th2 promoting activities and induces an a nonpolarized cellular immunity. These findings reinforce our previous studies indicating that TLR agonists could be potentially used in formulations of vaccines against allergy.^{10,11,16,26} However, it should be noted that depending on the allergen and the TLRs agonists used, different outcomes might occur. For instance, we showed previously that mice sensitized with *B. tropicalis*/Alum plus bacterial endotoxin developed airway inflam-

mation dominated by neutrophils upon *B. tropicalis* challenges, which was associated with immune-deviation toward Th1/Th17 pattern.⁹ We suggested that this type of immune-deviation occurred because commercial endotoxin activated simultaneously TLR4 and TLR2.⁹ This dual activation of TLR4 and TLR2 lead to Th17 immunity and neutrophilic dominated inflammation via MyD88 adaptor molecule.⁹ However, as shown here and similarly to what we found using the OVA-model, CpG dampened Th2-mediated responses indicating that TLR9 signaling in WT B6 mice is essentially inhibitory of Th2 sensitization. These results demonstrate that CpG has a potent anti-allergic activity to OVA, a food allergen, as well as to *B. tropicalis* extract, a relevant source of aeroallergens. Therefore, it is not surprising that subcutaneous administration of a ragweed-TLR9 agonist vaccine was clinically effective in allergic rhinitis treatment²⁷ or that a novel TLR9 agonist showed clinical efficacy in persistent allergic asthma.²⁸ In

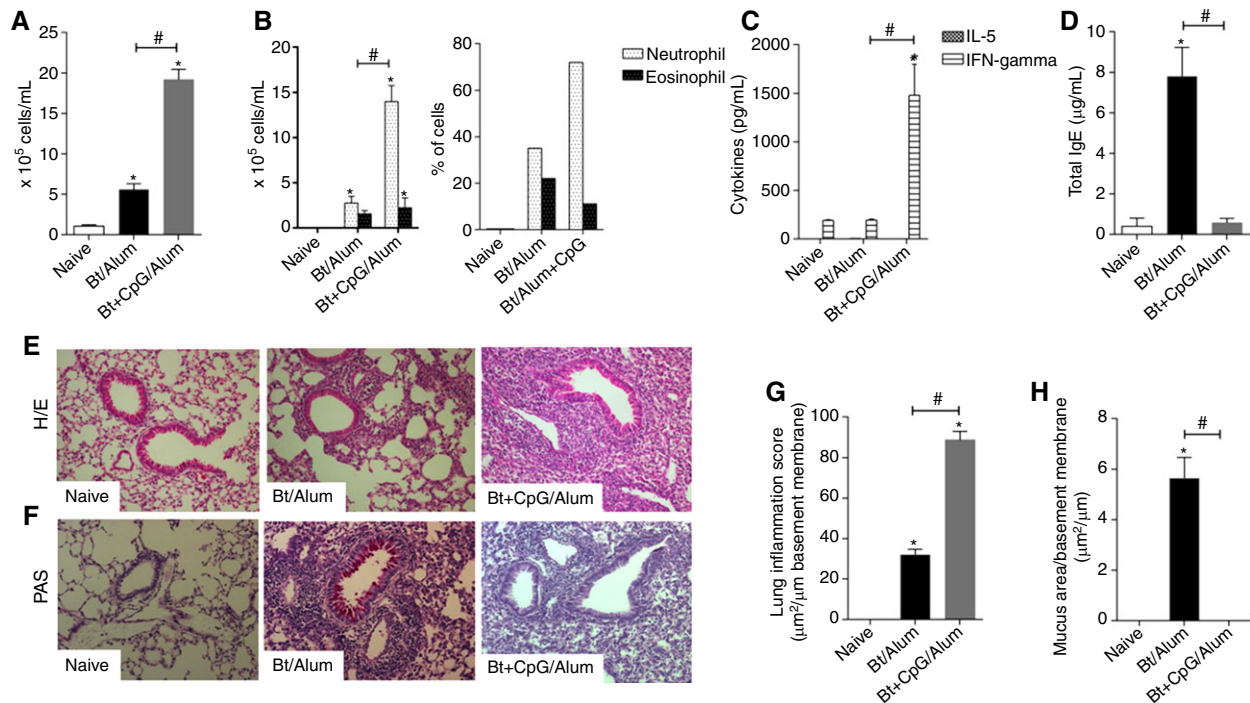


FIGURE 7 IL-10 is fundamental to prevent *Blomia tropicalis*-mediated Th1 immunity induced by sensitization with Alum/CpG adjuvants. IL-10-deficient mice on C57BL/6 background were s.c. sensitized with *Blomia tropicalis*/Alum or with *Blomia tropicalis*+CpG/Alum on days 0 and 7 and challenged i.n. with *Blomia tropicalis* on days 14 and 21. Samples were obtained on day 22. (A) Total cell, (B) differential cell counts, and (C) INF- γ in the BAL, (D) Total IgE in serum. Representative microphotographs of lung sections stained with H&E (E) and PAS (F), lung inflammation score (G), and mucus area/basement membrane score (H). Naive group consisted of non-manipulated animals. Results are expressed as mean $\pm$ SE of 3–5 mice per group and are representative of 2 experiments. One-way ANOVA * $P < 0.05$ different from Naive group; # $P < 0.05$ different from *Blomia tropicalis*/Alum group

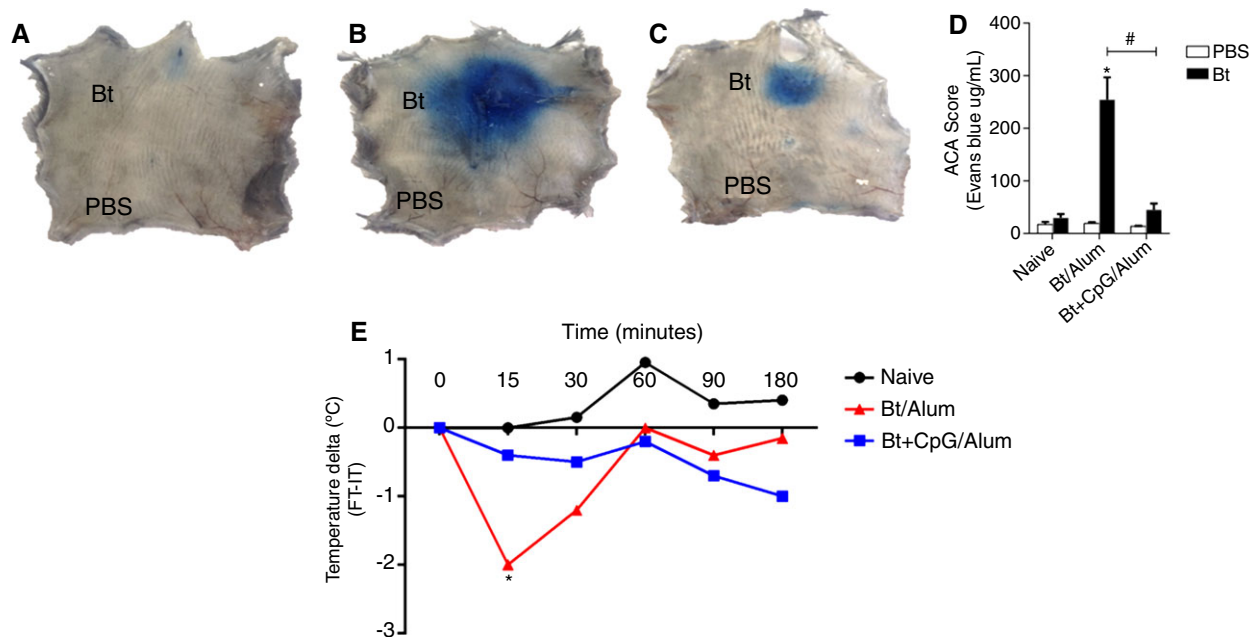


FIGURE 8 CpG impairs the development of *Blomia tropicalis*-specific active anaphylaxis reactions. WT C57BL/6 mice were sensitized s.c. with *Blomia tropicalis*/Alum or with *Blomia tropicalis*+CpG/Alum on days 0 and 7 and challenged with intradermal or intravenous OVA on day 21 to determine respectively active cutaneous anaphylaxis or systemic anaphylaxis. Representative pictures of skin active cutaneous anaphylaxis in (A) Naive, (B) *Blomia tropicalis*/Alum, and (C) *Blomia tropicalis*+CpG/Alum. (D) Active cutaneous anaphylaxis score, (E) Active systemic anaphylaxis measured by body temperature drop. Naive group consisted of non-manipulated animals. Results are expressed as mean $\pm$ SE of 3–5 mice per group and are representative of 2 experiments. One-way ANOVA * $P < 0.05$ different from Naive group; # $P < 0.05$ different from *Blomia tropicalis*/Alum group

summary, our work with CpG highlights its anti-allergic effects that could be explored in vaccine preparations using alum or immunotherapies against established allergies using specific allergens plus CpG.

AUTHORSHIP

F.P.B.N., R.W.A.C., E.G., N.H.Y., and D.M.F. performed the experiments and data analyses. F.P.B.N., D.M.F., A.L.R., and M.R. contributed in experimental design. F.P.B.N., R.W.A.C., D.M.F., and M.R. contributed writing and revising the manuscript.

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DISCLOSURE

The authors have no conflicts of interest to declare.

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SUPPORTING INFORMATION

Additional information may be found online in the Supporting Information section at the end of the article.

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